379-B-12

D 15.13:87-174

DNA-TR-87-174

RADIATION-INDUCED GERM CELL MUTATIONS—THEIR DETECTION AND MODIFICATION

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30 June 1987

Technical Report

CONTRACT No. DNA 001-84-C-0170

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UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b RESTRICTIVE MARKINGS
2a SECURITY CLASSIFICATION AUTHORITY N/A since Unclassified	3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited.
2b DECLASSIFICATION/DOWNGRADING SCHEDULE N/A since Unclassified	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)	5 MONITORING ORGANIZATION REPORT NUMBER(S) DNA-TR-87-174
6a NAME OF PERFORMING ORGANIZATION University of California at San Francisco 6b OFFICE SYMBOL (If applicable)	7a NAME OF MONITORING ORGANIZATION Director Defense Nuclear Agency
6c ADDRESS (City, State, and ZIP Code) Office of Contracts and Grants 145 Irving Street San Francisco, CA 94143	7b ADDRESS (City, State, and ZIP Code) Washington, DC 20305-1000
8a NAME OF FUNDING/SPONSORING ORGANIZATION 8b OFFICE SYMBOL (If applicable) MRCO/Harrison	9 PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DNA 001-84-C-0170
8c ADDRESS (City, State, and ZIP Code)	10 SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO: 62715H; PROJECT NO: U99QMXM; TASK NO: K; WORK UNIT ACCESSION NO: DH008302

11 TITLE (Include Security Classification)
RADIATION-INDUCED GERM CELL MUTATIONS—THEIR DETECTION AND MODIFICATION

12 PERSONAL AUTHOR(S)
Goldstein, Lawrence S.

13a TYPE OF REPORT	13b TIME COVERED	14 DATE OF REPORT (Year Month Day)	15 PAGE COUNT
Technical	FROM 830401 TO 870630	870630	70

16 SUPPLEMENTARY NOTATION
This work was sponsored by the Defense Nuclear Agency under RDT&E RMSS Code B350084466 U99QMXMK00093 H2590D.

17 COSATI CODES			18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Radiation; Dose Modification; Germ Cells; Risk Estimation; Mutagenesis
6	7		
6	5		

19 ABSTRACT (Continue on reverse if necessary and identify by block number)

Mutagenesis by densely ionizing radiation represents a significant long-term hazard to the individual. Few data are available to describe the dose-response aspect of mutation induction by either accelerated charged particles or fission spectrum neutrons in mammalian germ cells. To address this question, an in vitro assay of dominant lethal mutations was undertaken for animals exposed to either of these radiation sources. Special emphasis was placed on the action in spermatogonial stem cells. The data indicate that mutations were induced as a linear function of dose and there was no evidence of a threshold. The mutation rate was about 0.1 per Gy. Mutagenesis was also detected in females given x-radiation. The induction curve had an initial steep linear aspect and then a less steep slope. As with male germ cells, there was no evidence of a threshold. The in vitro dominant lethal data were compared to various cytogenetic assays and were found to be more sensitive indicators of mutation. In preliminary studies, the rate of stable mutagenic damage appeared to be modifiable by pretreatment with chemotherapeutic drugs. Because the radiation

20 DISTRIBUTION/AVAILABILITY OF ABSTRACT	21 ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	UNCLASSIFIED	
22a NAME OF RESPONSIBLE INDIVIDUAL Sandra E. Young	22b TELEPHONE (Include Area Code) (202)325-7042	22c OFFICE SYMBOL DNA/CSTI

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

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19. ABSTRACT (Continued)

sources chosen for this study are thought to be the most efficient for mutation induction, the values obtained may describe the upper limits of exposure for this endpoint. As applied to risk estimation, these data should be considered relative to the normal risk encountered and accepted by the population at large.

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SUMMARY

A long term consequence of exposure to ionizing radiation is the induction of stable mutations in germ cells. The frequency of radiation-induced damage in germ cells depends on the dose of radiation, the dose rate, the quality and the presence of chemical modifiers. The hazard presented to the zygote may be evaluated in terms of induction parameters as well as transmission parameters that include the probability that a damaged germ cell will complete its maturation and be capable of participating in fertilization.

Radiation induces damage in the form of intragenic mutations, structural chromosome aberrations and numerical chromosome aberrations. The aberrations are induced with a higher frequency than are intragenic mutations and therefore represent a class of significant importance. Phenotypically, zygotes with chromosome aberrations may be inviable or viable though developmentally compromised. Often they present a broad spectrum of clinical syndromes, some mild, some severe.

An in vitro - in vivo system using rodents has been developed to investigate the induction and transmission of chromosome aberrations in germ cells. Zygotes from an irradiated parent are grown in vitro to an early post-implantation stage and the frequency of successful development to defined morphological stages of development is related to the frequency in embryos derived from untreated parents. By assessing only fertilized oocytes one eliminates indirect effects due to lack of fertilization which in turn is a measure of germ cell killing.

Using this protocol male mice were irradiated with graded doses of fission spectrum neutron or accelerated argon nuclei. The dose response curve for each spermatogenic stage for mutagenesis was evaluated and the rate of mutation induct per unit absorbed dose was determined. For all stages, the rate of induction could be adequately described by a linear function suggesting there was no threshold. In other experiments males were pretreated with drugs which were thought to modify the mutational yield due to x-radiation. Preliminary evidence suggests that fewer mutations were produced in males given the pretreatments. The rate of mutation induction by x-radiation in oocytes was also determined and found also to be linear with induction kinetics that were characteristic of post-meiotic male germ cells. Cytogenetic assays were found to be not as sensitive as the mutational assay.

Since mutagenesis and carcinogenesis are most likely caused by the same or similar biological mechanisms, these data suggest that incidental exposure of personnel to densely ionizing radiation should be carefully monitored and that the long term effects of such exposure may not be manifest for long times. It is also understood that one cannot directly attribute a specific mutagenic or carcinogenic episode to such exposure.

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PREFACE

ANIMAL EXPERIMENTATION: Research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animals", prepared by the Institute of Laboratory Animal Resources, National Research Council.

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CONVERSION TABLE

Conversion factors for U.S. Customary to metric (SI) units of measurement for measurements used in this report

MULTIPLY → TO GET ←	BY → BY ←	TO GET ← DIVIDE
inch	2. 540 000 X E -2	meter (m)
joule/Kilogram (J/Kg) (radiation dose absorbed)	1. 000 000	Gray (Gy)
micron	1. 000 000 X E -6	meter (m)
rad (radiation dose absorbed)	1. 000 000 X E -2	Gray (Gy)
roentgen	2. 579 760 X E -4	coulomb/Kilogram (C/Kg)

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SECTION 1
INTRODUCTION

1.1 RADIATION-INDUCED MUTAGENESIS, CARCINOGENESIS, AND CLASTOGENESIS IN HUMANS.

1.1.1 Background.

The observation that patients exposed to Roentgen's beam and especially the clinicians using it developed skin ulceration and squamous cell carcinomas in areas exposed to the beam is among the earliest observations that radiation is carcinogenic. Soon after, the induction of mutations in a model experimental organism, the fruit fly was reported and the mutagenic aspects of radiation were recognised. A considerable effort in the 50 years since has defined the identity "mutagens are carcinogens". Since the cellular and molecular mechanisms of these processes are the same, both terms will be used in this report and discussion of one will be applicable to the other.

An understanding of the variables that affect the yield of induced mutants with both densely ionizing and sparsely ionizing radiation has significance in both the military and civilian arenas. Mutation induction in germ cells represents a significant long-term hazard for an individual or population exposed to ionizing radiation.

In considering the mutagenic/carcinogenic hazards of ionizing radiation it is important to understand that radiation-induced mutagenesis is a stochastic process. The probability of mutation is related to the dose. That exact relationship depends on many factors and even for a given regimen of exposure the probability of mutation induction at low doses is equivocal. Despite these limitations, it is assumed that each ionizing event has a finite probability of causing mutation. Thus, within limits, the number of mutations induced by a low dose of radiation in many individuals will be the same as the number of mutations induced in fewer individuals given a larger dose. Obviously the distribution within the population for these two scenarios will be very different.

Experimentally we must rely on extrapolations of results from high dose exposures of a few individuals in order to establish safe low-dose limits. A

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complete description of the biological and physical modifiers of radiation-induced mutations is beyond the scope of this report and has been the subject of extensive efforts by both national and international agencies (1,2). The studies described herein are concerned with quantitating the mutagenic actions and efforts to modify the response to three types of radiations in males and females. In order to put the data into perspective, it is necessary to introduce both general concepts of radiation-induced mutagenesis and this experimenter's own bias and interpretations.

1.1.2 Radiation-induced Clastogenesis.

The formation of chromosome aberrations (clastogenesis) after exposure to ionizing radiation was recognized early in the disciplined study of radiobiology and served as a basis for a mathematical model of radiation effects still in use today. The spectrum of damage to chromosomes is well documented. It falls into two main categories: (1) structural aberrations including chromosome aberrations which result when an unreplicated chromosome is damaged or chromatid aberrations when one replicated chromatid is damaged, and, (2) numerical aberrations which affect ploidy by causing the loss or addition of entire chromosomes.

Structural chromosome aberrations have been related to a variety of syndromes and many of these are associated with cancer or a predisposition to cancer. A 92% correlation between chronic myelogenous leukemia and the Philadelphia chromosome (3) has been found. Chromosomal aberrations are found in about half the cases of acute leukemia. Burkitt's lymphoma, Hodgkin's and non Hodgkin's lymphoma and lymphoblastic leukemia have also been associated with the presence of chromosome aberrations (for review see 11). The relationship of neoplasia to chromosome aberrations and the possible interaction of chromosome aberrations and oncogenes has been reviewed (4).

The few data on humans indicate that radiation can increase the frequency of chromosome aberrations in germ cells. In a multivariant analysis Alberman (5) found that mothers of chromosomally abnormal fetuses had received a larger mean gonadal dose of radiation than did matched cohorts. A higher frequency of chromosomally abnormal fetuses were sired by men who had received occupational exposures (6). Brewen et al (7) found overall induction rates of 4.0-7.1% for translocations in spermatocytes of men given 78 to 200 R of photons. However, neither chromosome aberrations (8) nor increased mortality rates (9) were found in

children of A-bomb survivors. Enhanced aberration frequencies were found in the lymphocytes of nuclear shipyard workers (10) and workers in nuclear power plants (11) but not in American uranium miners (12).

Most numerical aberrations result in the death of the zygote before or soon after implantation. Boue et. al (13) estimate that 66% of all early abortuses have chromosomal anomalies, and the uncertainty in recognizing an early pregnancy may make this an underestimation. Some chromosomally aberrant babies survive to term. Trisomy 21 (Down's Syndrome) is probably the autosomal aneuploidy with the longest life expectancy. Individuals with trisomy 18 (Turner's Syndrome), trisomy 13 (Pateau's Syndrome), or trisomy 8, all demonstrate severe neurological disorders and require extensive post-partum maintenance. Sex chromosome anomalies are more prevalent but usually do not show severe clinical symptoms.

1.2 EXPERIMENTAL SYSTEMS FOR THE STUDY OF RADIATION-INDUCED GENETIC EFFECTS.

1.2.1 Background.

Germ cell or somatic mutation induced by ionizing radiation, whether by biomedical application or environmental contamination, is characteristic of low to moderate exposure levels. Since it is prohibitively expensive to identify rare events in whole animals, systems to evaluate the mutational yield rely on extrapolating the results from relatively high doses administered to relatively few individuals. This requires an understanding of the induction kinetics and therefore data must be accumulated at several doses.

The doubling dose (the dose to induce twice the background mutation frequency) for a variety of genetic endpoints ranges from 16-51 R of photons (14). There is evidence to suggest that densely ionizing radiation is much more efficient than photons (1,2). However, less is known about mutagenesis by densely ionizing radiation since many physical factors (dose, dose rate, ionization density) have large impacts on the mutation rate. In the absence of data to the contrary, the induction curve for neutron-induced mutagenesis is described as a linear function of dose although there is considerable uncertainty as to the slope. Almost all our understanding of mutagenesis by densely-ionizing radiation is based on exposures to fission-spectrum neutrons and even these data include a non-quantified contribution

due to gamma radiation contamination.

Most of the data upon which safe exposure limits are based are from the mouse specific-locus mutation test used extensively by Russell in this country and Searle in England (for review see 15). Data from these laboratories indicate that for low LET photon irradiation the mutation rate (mutants per rad) is less at very low (<300 rad) and very high (>600 rad) exposures than for exposures between 300 and 600 rad, and that the mutation rate is greatly reduced when the dose rate is decreased or the total dose is divided into many small increments. These observations have been interpreted to signify that specific-locus mutations are induced by both single-hit and two-hit processes (16), and that the cell can repair a significant amount of premutational, one-hit lesions (16,17). Using these assumptions, mutation induction by photons is a curvilinear function of the dose.

There are variations of the basic specific-locus test. Kohn used electrophoretic changes in the histocompatibility complex to increase the sensitivity of the test by enrolling more genes (approximately 35) to the analysis (18). Unfortunately, the assay is tedious and imprecise since small changes may not be detected. Ehling and his coworkers (19) have used the endpoint of cataract formation, a phenotype that may involve as many as 25 loci in humans (20). Several other genetic systems are available including the sex-chromosome-loss technique of L.B. Russell (21), the heritable translocation test of Generoso (22), and the dominant lethal assay first used by Snell (23) and modified for toxicological studies by Epstein (24). The first two assays detect aneuploidy and reciprocal translocation respectively; the third detects these two kinds of damage in addition to many other kinds of structural damage but cannot separate the relative contributions of each to mutagenesis.

The first genetic consequence of ionizing radiation in mammals was described by Snell (23) who found a reduction in the size of litters sired by male mice given X-radiation. This effect was heritable as semi-sterility in the viable offspring. Koller and Auerbach (25) then demonstrated that this reduced reproductive integrity was the result of radiation-induced chromosome aberrations in the germ cell. This genetic effect of radiation or other mutagens has been termed dominant lethal mutation. The broad range of chromosomal defects that can be detected by a dominant lethal assay corresponds well to the broad range of chromosomal defects

caused by ionizing radiations, and therefore the dominant lethal assay would appear to be the assay of choice when one is concerned with questions of mutant offspring from any of several types of lesions.

1.2.2 Radiation Effects in Male Germ Cells.

1.2.2.1 Anatomical and Cellular Considerations. The seminiferous epithelium of the testis normally contains germ cells in various stages of maturation. The stem cell spermatogonia, located at the basement membrane of the tubule, proliferate and either replenish the stem cell compartment or progress to a differentiative component (26-28). The precise cellular identity of the stem cell spermatogonium remains controversial. Intermediate spermatogonia form syncitia which then separate, undergo meiosis in the spermatocyte stage which differentiate to form spermatids. Spermatids undergo protamine-mediated nuclear condensation form a flagellum and become mature spermatozoa. The differentiation of spermatogonia to mature spermatozoa takes about five weeks in the mouse (29).

1.2.2.2 Germ Cell Lethality. Spermatogonia are represented by both radioresistant (A_{stem}) and radiosensitive (A_1,A_2,A_3,A_4, Type B) cells with LD_{50}s of 700 rad and about 25 rad (30) X-radiation respectively. Spermatocytes demonstrate an intermediate sensitivity and have a four-fold range from the most sensitive (Preleptotene, LD_{50} = 200 rad) and the most resistant (Diakenesis-Metaphase I, LD_{50} = 830 rad) (31). Both spermatozoa and spermatids are resistant to killing by ionizing radiation (31,32).

A dose of 600 rad X- or γ-radiation will therefore show no immediate effect on sperm count since the cells were exposed in the resistant spermatozoa or spermatid stages. Three weeks later, oligospermia is manifest as the few survivors of the germ cells exposed in the radiosensitive spermatocyte and spermatogonial stages reach the cauda epididymis. This transient sterility varies in duration and extent with the dose.

1.2.2.3 Genetic consequences. Radiation induced damage in germ cells is manifest by the zygote, which has a contribution from such cells as part of its genome. X-ray induced dominant lethal mutations are demonstrated in a higher frequency in fertilizations in which the germ cells were exposed after meiosis than in

fertilizations where germ cells were exposed before meiosis (33). This period is also characterized by a high yield of chromosome damage in germ cells (34) without concomitant oligospermia (35). In vivo analysis of dominant lethal mutations induced in spermatocytes and spermatogonia type B is somewhat inaccurate since these stages are sensitive to cell killing. Fertilization failures reflecting cell killing are not easily distinguished from dominant lethal mutations expressed before implantation (33). Spermatids appear to be more sensitive than spermatozoa to dominant lethal mutation induction by ionizing radiation (33,36). The long-term genetic consequences for mutations of post-meiotic germ cells is important as an indicator of the risks posed by chronic radiation.

Dominant lethal mutations in spermatogonia are expressed as "small moles" and represent early post-implantation arrests (37). Significant pre-implantation deaths, measured as the number of corpora lutea not represented by an implant are also found. These forms are found after X-ray doses of 600 rad (38).

1.2.2.4 Cytogenetic Consequences. Structural aberrations of both the chromosome-type (terminal deletions, minutes, acentric or centric rings, dicentrics, reciprocal translocations, inversions, etc.) and the chromatid-type (terminal deletions, chromatid minutes, isochromatid deletions, interarm interchanges, etc.) are found in irradiated germ cells. Numerical aberrations (aneuploidy) are also detected. Both types of aberrations can have untoward consequences since viable offspring with such defects are produced.

By far the most frequent form of chromosome aberration induced in spermatogonia is the Ring-of-Four (R_{IV}), accounting for approximately 70% of all aberrations; the Chain-of-Four (C_{IV}) accounts for about 30% and less than 1% are trivalents + univalents (39). The yield increases linearly with X-ray doses to 600 rad but then decreases with increasing doses (40-43). This humped dose-response curve has been interpreted to reflect the selection at meiosis of germ cells having an induced translocation (44). The induction of translocations by neutrons also demonstrates this type of curve with a maximum rate of induction at 150 rad (45,46). The RBE for translocation varies with the dose rate (45,47) and as the ionization density increases up to approximately 100 keV/μm (48,49). The RBE can reach a maximum of more than 20 in mammalian cells (1,2) and can approach 100 when measured in plants (50,51).

Less is known about translocation induction in post-meiotic stages than in pre-meiotic stages. Brewen (52) and others (53) have introduced an assay in which damage to the male pronucleus is analyzed in fertilized eggs. These have been used to assay damage by methyl methanesulfonate in spermatozoa and spermatids. Other systems using sex chromosome loss (21) or dominant lethal mutations (22,23) indicate that the post-meiotic stages are more sensitive to the induction and transmission of translocations.

Variables other than the quality of radiation can influen the rate of translocation induction. Significant differences when the two testes of the same mouse have been irradiated may reflect selectivity in spermatogonial repopulation (54). The yield of aberrations also varies with time after irradiation, reaching a peak at 13-18 weeks (55). Although laboratory strains of mice are remarkably consistent in the sensitivity to radiation-induced translocations (40), they are 3-4 fold more sensitive than are wild mice (56). This difference cannot be caused by differences in the background rate since this frequency is very low for both populations

1.2.3. Radiation Effects in Female Germ Cells.

1.2.3.1 Anatomical and Cytological Aspects. In the female mouse, mitotic divisions of primordial germ cells form oogonia by days 9-12 of embryogenesis (57). At this time some oogonia enter meiosis while the majority degenerate (58). A second wave of atresia occurs on days 14-18 of embryonic life (57,59) and soon after birth, all germ cells are in the dictyate (resting) stage. Since all oogonia have degenerated, no renewal of the oocyte population is possible. The female mouse has a maximum of about 900 oocytes three days after birth after which they steadily decline by random atresia and ovulation (56). Most germ cells in the mature female are in the resting stage and a few have resumed meiosis just prior to ovulation.

The oocytes become surrounded by layers of granular cells (59) and microscopic examination of the ovary reveals oocytes with a single layer of follicle cells (primary follicles), oocytes surrounded by multiple layers of follicle cells (intermediate follicles) and oocytes in follicles having an antrum (mature follicles). Maturation of the oocyte from the dictyate stage to Metaphase II is hormonally controlled. Edwards and Gates (60) have timed the sequence of oocyte maturation following induction by exogenous hormonal stimulation and found that some oocytes

resume meiosis and progress to Metaphase I 4.5 hours following injection of follicle stimulating hormone; by 12-14 hours, the oocytes are in Metaphase II and are ready to be ovulated.

1.2.3.2 Radiation-Induced Cell Killing. Female germ cells in the mouse show a wide range of sensitivities to the lethal effects of ionizing radiations. Only 1-9% of primordial oocytes survive an X-ray dose of 20 rad (61) and the LD_{50} for this cell type is approximately 8 rad (62). It is reasonable that even a small dose of ionizing radiation will restrict fertility to those oocytes exposed while in a mature follicle, or to about four litters, since more than 99.9% of primordial oocytes are killed (62). In contrast, dictyate oocytes in mature follicles are radioresistant and have an LD_{50} of about 700 rad X-radiation (62,63). Oocytes in metaphase I have intermediate radiosensitivity with an LD_{50} of 50 rad (62-64).

1.2.3.3 Mutagenesis. Since the primordial oocyte is very sensitive to killing by X-radiation (59-61) studies of dominant lethal mutations in females have been restricted to oocytes in the dictyate or maturation stages. Edwards and Searle (65), using the techniques of artificially induced and timed ovulation (60) found a peak of X-ray-induced dominant lethal mutations between metaphase I and metaphase II. This finding is in substantial agreement with that of Russell and Russell described in naturally ovulating mice (64). Searle and Beechey (65) tried to establish the RBE for neutrons in oocytes by measuring both the induced translocation rate at metaphase I and by fertility testing of f_1 offspring but their estimate of 3.0 was qualified by the small sample size. Since removal from the maternal environment does not effect the rate of induction of dominant lethal mutation or their expression (66-68), it would seem reasonable that the technique described by Brewen et al (52,69) for mass isolation of oocytes or one-cell stage embryos is particularly suited to investigations of dominant lethal mutations.

1.2.3.4 Clastogenesis. Studies which analyze the cytological effects of radiation on oocytes have been facilitated by techniques for collecting large numbers of oocytes. These techniques include superovulation and colchicine treatment of ova (52,69). Some data collected for dictyate oocytes in various stages of follicle development and analyzed at Metaphase I indicate a peak of induced chromosome aberrations for oocytes X-irradiated 14 days before metaphase I (70-72). The dose that gives an average of 1 aberration in 50% of the cells is about 400 rad (70).

Since the mature germ cells are in the dictyate stage in the mouse it would be expected that all aberrations would be of the chromatid type. Brewen et al (69) did find chromatid interchanges as predicted, and this is in substantial agreement with other workers who found this type as well as isochromatid deletions and chromatid deletions (70-72). However, there were also quadrivalent chromosome aberrations of the RIV and CIV forms (70), and these types were not expected for chromatid aberrations.

The number of aberrations found in oocytes during maturation varies greatly with the stage irradiated (71,72).

1.3 ASPECTS THAT MODIFY THE MUTATIONAL YIELD.

Any study that purports to demonstrate the rate of mutation for radiation is woefully uninformed about the many variables that can influence the outcome. Of the many physical and chemical aspects that comprise the complex interactions that ultimately result in a detectable mutagenic event, this report will consider only four: dose, dose rate (or interval between doses), radiation quality, and chemical modifiers.

1.3.1 Effect of dose on mutation rate.

In all systems so far investigated, the mutation rate initially increases with increasing dose but then levels off before decreasing at higher doses. This humped-shaped induction curve is thought to reflect the killing of mutated cells at the higher doses. A corollary to this interpretation is that mutations are induced with an efficiency that is higher than that for the induction of lethal lesions. The inflection point in the dose response curve for photon-induced specific locus mutations occurs at about 600 R (13).

The exact shape of the initial or rising portion of the dose-response curve is still controversial. For the purpose of establishing exposure limits, the shape is taken to be linear and without threshold. Russell (73) has argued that the shape of the curve is curvilinear and consistent with mutations involving discrete regions of specific genes and therefore as being true point mutations. Others (16,74) have interpreted the same data as reflecting induction by both single hit and two hit mechanisms and therefore a curvilinear induction consistent with events at the chromosomal level is proposed. This debate is academic when considering effects

due to densely ionizing radiation which is considered to act essentially by one-hit mechanisms.

In general mutations are induced by densely-ionizing radiation as a linear function of dose and therefore with single hit kinetics. Thus, there really is no safe exposure limit and one must talk in terms of acceptable risk. While exposure limits for radiation workers are often set with a zero incidence bias, it is the opinion of this investigator that meeting such criteria are of limited usefulness. One must evaluate in terms of acceptable risk and thus decide if a casual involuntary exposure exceeds the risk associated with normal activity.

1.3.2 Dose Rate.

The radiation induced mutant rate is a function of the dose rate at which the radiation is delivered. Since civilian and military exposures may be chronic, acute, or somewhere in between, the impact of dose rate on the final mutant yield is important.

For mutagenesis by photons, the mutant rate decreases with decreasing dose rate. The relationship may be due to the interval between doses rather than the dose rate since fractionation is the usual method by which the dose rate is reduced in model systems. These results have been interpreted to mean that some mutational damage is repaired within the cell and that when high total doses are given at low dose rates the repair system becomes saturated and the rate of mutagenesis increases. Others believe that this increase merely reflects the fact that some mutations by photons results from two-hit processes at the chromosomal level and the increase reflects the stochastic processes involved.

The dose rate phenomenon again demonstrates the futility of attempting to establish the mutation rate for a given radiation exposure. For setting risk limits, it is prudent to use the "worst case scenario" and therefore the rate resulting from exposure at high dose rates.

The picture is not simple for densely ionizing radiation either. The amount of recovery is a function of the ionization density although the recovery between doses is in general not as pronounced as for photons. This parallels the phenomenon noted for cell killing in normal tissues and tumors. The relationship of recovered dose per fraction as a function of ionization density is best determined in beams of

defined Linear Energy Transfer (LET). Because neutrons have a broad distribution of LET (75) in addition to variable amounts of gamma ray contamination, it is not a simple matter to define the mutation effect at low doses. However, there is a well established phenomenon known as the "reverse dose rate effect" that has been described in specific locus mutation experiments in mice given chronic fission spectrum neutrons (76). The mutant rate under these conditions exceeded the rate following acute exposures and therefore it was concluded that such exposures represented a higher risk than acute exposure. Recent results with other specific locus tests indicate again that the results are not easily interpreted since the mutation rate in different genetic loci were different (19).

1.3.3 Radiation Quality.

It has been established that the mutant rate per unit absorbed dose is higher for densely ionizing radiation than for sparsely ionizing radiation. Effectiveness factors range from a few percent to 50-fold increases. The caveats mentioned above also hold for the impact of radiation quality and for this reason it is imperative that one address this aspect in matched studies where the dose and dose rate are the same. In general, fission spectrum neutrons are assigned the highest effectiveness factors of any radiation source (1,2). A review of the literature indicates however that few other sources were available for comparison and that this conclusion in whole animals is based on comparisons between fission spectrum neutrons and accelerated neutrons with mean energy greater than 2.0 MeV (15). In work done in cells irradiated with accelerated charged particles indicates that the efficiency of mutation induction increases with increasing ionization density up to about 95 keV/um and then decreases at higher ionization densities (48). The relationship of mutation induction to ionization density appear similar to that reported for cell killing.

No data are available that relate the mutant rate at a given ionization density as a function of dose rate. A reverse dose rate effect has been described for densely ionizing radiation for cell killing after fractionated exposures (77,78,) and it is reasonable to assume that because at least some of the mechanisms of cell killing and mutation induction are similar, that a reverse dose rate effect for fractionated exposures to charged particles will also be found.

1.3.4 Chemical Protectors.

The few data available for mutation induction in the whole animal previously treated with sulphydryl containing radioprotectors suggest that there is dose modification (79). In the clinic a protection of another type has been described in patients given alternating schedules of radiation and drugs, especially actinomycin D (80). In such patients the rate of induction of secondary neoplasms appears lower than in patients given radiation alone. A similar phenomenon has been described in cell culture and is known as "mutational extinction" (81). Clearly the ability to protect populations from the genetic risks of ionizing radiation has far-reaching implications and is the subject of intensive study.

Although the protection of the genome to mutations induced by densely ionizing radiation through chemical intervention has been reported, considerably more work is needed before definitive statements about this activity can be made.

1.4 OVERALL PROGRAM OBJECTIVES.

The studies to be described in this contract proposal are designed to: (1) evaluate the mutagenic hazards to male and female germ cells caused by densely-ionizing radiations, (2) gain insight into the mechanisms responsible for mutation induction, and (3) attempt to modify the hazards of mutagenesis in germ cells by applications of drugs and other dose-modifying procedures.

1.4.1 Specific Aim: to determine the genetic effects of 95 keV/μm charged particle irradiation of male germ cells.

Both genetic (82-85) and cytogenetic (48,86,87) studies indicate that ionizing radiation with an LET of approximately 100 keV/μm is the most efficient for mutation induction. Analysis of this type of radiation in male germ cells has been hampered by the inability of particle accelerators to impart sufficient momentum to large, charged nuclei such that the particle can traverse a cm or more of tissue. Advances in accelerator technology at the Lawrence Berkeley Laboratory now make such accelerations routine. A beam of partially stripped Argon nuclei in an unmodified configuration has the necessary LET in the entry (plateau) region and is amenable to such investigations. Previous studies were performed at ionization densities above and below 95 keV/μm, and it is anticipated that irradiations in the

plateau region of the Argon beam will result in RBE ratios for mutagenesis that are higher than those of the other sources. Elucidation of the mutagenicity of this source may therefore act as an upper limit for radiation mutagenesis. Establishing the mutagenic hazard of this source will fill in an essential point in the RBE-LET relationship such that a meaningful estimate of mutagenic hazard (with respect to intergenic lesions) may be made for radiation sources of definable LET. Defining the overall curve for mutant yield as a function of dose will also enhance our understanding of the mechanism of induction and the relative contribution of single-hit and two-hit mechanisms.

1.4.2 Specific Aim: To determine the genetic effects of fission spectrum neutron irradiation of male germ cells.

Since fission spectrum neutrons are usually assigned the highest RBE of any external radiation (1,2,51) information that defines the dose-response relationship and can contribute to an understanding of the mechanisms involved and will have far-reaching significance to many defense-related activities. Especially important is defining the dose-rate kinetics at low doses since this source exhibits a "reversed dose rate effect" (i.e., the mutation yield for a dose given acutely) and this may be reflected in the shape of the mutation induction curve at low doses. The acute studies proposed here are intended to fill a gap in our overall understanding of mutation induction and provide a baseline for further studies with chronic irradiations.

1.4.3 Specific aim: To determine the genetic effects of x-irradiation of murine oocytes exposed in a radioresistant phase of their maturation.

Human oocytes are arrested in the radioresistant diakinesis stage of meiosis whereas the mature oocyte of the mouse is in the radiosensitive dictyate stage (88). However, murine oocytes ovulated within 9.5 days of treatment show an increased radiation-induced translocation frequency at metaphase I (69-72), and Russell (89) has argued that these stages are useful in predicting the response of the human arrested oocyte if one assumes that the human oocyte is at least as sensitive as the most sensitive oocyte stage of the mouse. Although the kinetics of translocation

induction is consistent with a 2-hit mechanism for photons, for risk assessment a linear relationship should be assumed. A more prudent estimate (based on specific locus mutations) may be that the human oocyte is about half as sensitive to mutation induction as the spermatogonium (89), but this should be derived experimentally. An understanding of the nature of induced intergenic lesions will also be valuable in ascertaining the relative risk posed by occupational exposure and its relationship to high incidences of "spontaneous" abortions.

1.4.4 Specific aim: To modify the genetic effects of photon or densely ionizing radiation by chemical intervention.

Attempts to modify the rate of radiation induced mutations by administration of radioprotective compounds have been reported for over 20 years. Using 2-aminoethylisothiuroniumbromide hydrobromide (AET), Leonard could reduce the radiation induced dominant lethal mutant rate in spermatids but not other germ cell stages (78). They could not affect translocation yields in treated spermatogonia (90). Modifying the mutant rate using "non-cross reactive" drugs such as vincristine and mechlorethamine have not been attempted although reductions in the incidence of second neoplasias in patients treated with certain chemotherapeutic drugs and radiation have been reported. Initial studies will attempt to modify the mutant rate by chemotherapeutic drugs with known cellular effects. Another strategy is to use sulphydryl-containing compounds that have been shown to modify the mutant rate in cultured cells irradiated with photons or neutrons.

SECTION 2
MATERIALS AND METHODS

2.1 RADIATION SOURCES, SETUPS AND DOSIMETRY.

2.1.1 X-radiation.

A Westinghouse Quadroconex X-ray machine, located at the Radiation Oncology Research Laboratory of the Department of Radiation Oncology of the University of California San Francisco was used. The unit was operated at 250kVp, 15 ma with 1.5mm Cu and 0.5mm Al filtration.

The mice were placed in lucite holders on a rotating turntable at a distance of 100 cm from the source. Dosimetry was by LiF thermoluminescent dosimeters that were imbedded in tissue phantoms inside the radiation jig and irradiated in this geometry. The dose rate measured by this procedure was 217 cGy min^{-1} in this geometry.

2.1.2 Accelerated Charged Particles.

Accelerated charged particles were delivered by the Bevalac Accelerator of the Lawrence Berkeley Laboratory of the University of California, Berkeley. The Bevalac consists of two accelerators operated in series. Partially-stripped atoms are injected into the Heavy-ion Linear Accelerator (Super Hilac) where they are accelerated. The beam is then transported via vacuum tubes with bending and focusing magnets to the injector port of the Bevatron Synchrocyclotron where the beam is accelerated again. The beam is extracted and delivered into the Biomedical Cave of the Bevatron facility.

The shape of the beam is refined with brass absorbers and the residual range reduced (if necessary) by a variable thickness water absorber. The beam also passes through thin wire ionization chambers both upstream and downstream of the sample. For the experiments performed here no further beam modification was attempted but the facility has the capacity to degrade the Bragg peak region by sending the beam through a spiral brass ridge filter which acts as a second variable thickness absorber.

The system was operated in the RADSFAST mode which reads the accumulated dose in the upstream absorber and cuts off the beam within 1% of the dose requested. The dose is delivered in pulses every 6 seconds and the beam was attenuated to give an average dose rate in the range of 100 cGy min^{-1}. The beam geometry was established and the mice positioned in the entry region without water in the range shifter.

The mice were put in plastic tubes and positioned vertically within the beam. Each mouse was irradiated individually with a pelvic field shaped by cerrobend collimators placed directly before the holder. The mice were automatically positioned within the beam by a sample positioning device.

2.1.3 Fission Spectrum Neutrons.

Fission Spectrum neutrons were generated by the TRIGA water-cooled reactor located in the Department of Nuclear Engineering of the University of California, Berkeley. Irradiations were performed in collaboration with Dr. Tek Lim and his staff.

The mice were immobilized in a lucite and wood jig consisting of 30 chambers. Each chamber measured about 3.75 X 12 cm. The mouse could be immobilized without discomfort. The box was positioned vertically about one meter from the source. A 2.5cm-thick lead absorber was placed about 10 cm before the biological sample holder.

Actual doses were determined by aluminum foil dosimeters included at several locations during the exposure. Estimates of the times necessary to accumulate the total dose were made by 2 methods. In the first Dr. Lim and his staff performed a retrospective analysis based on their own experience by assigning an effectiveness ratio for this set-up. In the second, Dr. Clifton Ling of the Department of Radiation Oncology of the University of California San Francisco estimated the fraction of the total neutron dose accounted for by the 0.5 to 2.0 Mev Neutrons of interest. The Ling calculations are summarized:

ESTIMATES OF CONVERSION FACTOR FOR REACTOR NEUTRON - FLUX TO RAD/MIN

Assumptions: (1) neutron spectrum is that of U-235 fission*

(2) relative number of neutrons as function of assay given by ICRU Report #26, p. 55**

(3) Kerma factor adopted from ICRU #26, Appendix II

(4) Negligible correcting from Kerma to rad

E_u	N. Relative Number of neutrons	K H_{20} Kerma Factor ($x10^{-8}$)	N.K.
0.25	60	0.123	$7.2x10^{-8}$
0.75	69	0.21	14.5
1.25	58	0.28	16.2
1.75	48	0.32	15.4
2.25	35	0.34	11.9
2.75	27	0.37	10.0
3.25	20	0.43	8.6
3.75	15	0.45	6.8
4.25	11	0.46	5.1
4.75	8	0.48	3.8
5.25	6.1	0.49	3.0
5.75	4.5	0.50	2.3
	Σ361.6		Σ104.7

*Probably a large component of thermal and epithermal n's.

**This is the spontaneous fission spectrum. The measured data in Fig. 5B on p. 53 might be more appropriate.

Average $K = \frac{104.7 \times 10^{-8}}{361.6}$ rad.cm^2 $= 0.29 \times 10^{-8}$ rad.cm^2

If the neutron flux is F $\frac{1}{cm^2 . sec}$ then dose rate

$$d = 0.28 \times 10^{-8} \frac{\text{rad.cm}^2}{\text{min}} \times \frac{1}{2} \times \frac{\text{dose}}{\text{min}} = 17.4 \times 10^{-8} \frac{\text{rad}}{\text{min}} . F$$

$$D = 2.96 \frac{\text{rad}}{\text{min}}$$

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Both methods were within 5% of one another. During the actual irradiation, the reactor was powered up or down so that the total exposure time (but not the dose rate) was the same. It is not felt that the range of dose rates used will materially affect the results.

Since the animals had to be transported to the Berkeley Facility, it was necessary to subject the controls to a similar stress. Therefore, the controls were transported to and from Berkeley but they were not irradiated nor placed in the irradiation holder. Survival of irradiated animals was excellent however (one fatality in 220 exposed when the animal wedged himself under a partition in the chamber and suffocated). It is not felt that the animals were exposed to a stressful condition during the exposure.

2.2 BIOASSAY.

2.2.1 Induced ovulation and mating.

Female mice were hormonally stimulated to ovulate according to schedule. Two days before mating they were injected with Pregnant Mares' serum (PMS) which has follicle stimulating activity in the mouse. The dosage was adjusted periodically so that no more than 20 ova would be ovulated by each mouse since previous experience has indicated that the developmental capacity in vitro of the embryos is unpredictable when they are derived from high-number super ovulations. The dosage range for PMS was 1.25 to 5.0 IU delivered intraperitoneally. Immediately before mating (two days after the PMS) the mice were injected with human chorionic gonadotrophin (hCG) which has luteinizing activity in the mouse. The dose used was always 5.0 IU since the ovulatory response was found to be unaffected with changes above or below this level. Immediately after injection the females were paired with an irradiated or control male.

Matings were begun on the day of irradiation and continued twice per week for the next five weeks. Matings took place on day 0, 4, 7, 11, 14, 18, 21, 25, 28, 31 and 35. Thereafter the matings were performed once per week until ten or more samplings after day 35 were accrued.

Successful matings were detected by the presence of a copulatory plug. When the plug was not obvious visually, the vagina was probed gently for the plug.

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Plugged females were identified and enrolled in the assay. Those without a plug were grouped, held two weeks after which ovulation was again induced and they were given a second mating opportunity.

For matings with males irradiated with accelerated argon the females mating with males from each exposure group were caged together and held for further analysis. The same procedure was used when the female was irradiated and for matings with drug-treated males in the dose modification studies. For matings with neutron-irradiated males the females were kept overnight with the coded male and identified with his code.

2.2.2 Drug Delivery in treated males.

Males were injected with procarbazine and nitrogen mustard. The dose of procarbazine was 125 mg/kg and it was injected intraperitoneally. Nitrogen mustard (mechlorethamine) was administered at a dose of 2.25mg/kg delivered via the tail vein. A 4 hour interval was used between injections. The order of administration was either mechlorethamine first or procarbazine first. One week after the drug treatment, the mice were given 200 cGy of x-radiation delivered whole body. Five weeks after the irradiation, they were enrolled in the dominant lethal assay. Mice receiving drugs only (in both combinations) or X-radiation only served as controls. Other mice received no treatment.

2.2.3 Embryo Retrieval.

Females with copulatory plugs were killed by cervical dislocation one day after the plug was detected. A mid-ventral pelvic incision was made after the area was swabbed thoroughly with 70% ethanol. The oviducts were identified and removed by teasing away the adhering ovary and making a cut at a level just below the ovary and at the anterior of the uterus. The oviducts from each female were placed in individual drops of L-15 medium modified according to the procedure of Spindle and Pedersen (91). The oviducts were handled using sterile technique from the time of their removal.

If necessary the oviducts were rinsed with clean, modified L-15 and placed in drops of fresh medium. The anterior region of the oviduct was identified (ampulla) and grasped gently between the tines of a watchmaker's forceps. A 30Ga

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hypodermic needle whose point had been removed was inserted into the ampulla and grasped firmly with the forceps. Care was exercised not to disrupt the tissue when identifying and isolating the ampulla. The oviduct was gently irrigated by forcing some L-15 medium through it by a syringe attached to the 30Ga needle. During the irrigation the extrusion of squamous epithelial cells, ova and 2-cell stage embryos was noted from the cut uterine end. If no extruded material was noted the needle was withdrawn and the oviduct searched along its length for evidence of a tear. Once located, the needle was inserted upstream of the tear and the irrigation procedure was repeated. After flushing, the oviduct was removed from the drop of L-15 medium and discarded. These procedures were performed in a sterile petri dish (60mm) with the aid of a dissecting microscope at 15-30X.

All the oviducts from a treatment group were flushed at the same time. Typically with 3 people performing this, the total time of dissection and embryo removal was about 4 hours.

2.2.4 Embryo culture.

After removing the embryos and unfertilized ova from the oviduct, the embryos were counted and transferred to fresh drops of modified L-15 medium. Transfer was by a glass Pasteur pipet whose end had been drawn to a microtube. With experience the microtube that accommodated the embryos without including too much medium could be fashioned by eye. The number of embryos from each female was noted, as was the number of unfertilized or degenerating ova. Only embryos in the 2-cell (and occasionally the 4-cell) stage were transferred. After washing in the modified L-15 medium, the embryos were transferred to Ova Culture medium formulated after Biggers (92).

For studies involving the induction of dominant lethal mutations after irradiation of males with accelerated argon or for males given x-radiation after having been treated with drugs, the embryos were pooled into groups of about 150 but the fertilizations from a given female were not divided into more than one dish. For experiments describing the induction of dominant lethal mutations by X-rays in females or in males given fission spectrum neutrons, the embryos were not pooled but clutches from each female were cultured individually. The eggs were placed in 0.5ml of the modified Biggers medium contained in the central well of an Organ culture dish. The outer ring was filled with 1.5ml of sterile water. The paper

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absorbant material, if present initially, was discarded. The organ culture dishes were incubated in the dark at 37°C in an atmosphere of 5% CO_2 in air. Humidity was maintained at 92%. The embryos were incubated in this medium under these conditions for 3 days.

After 3 days the embryos were analyzed for the frequency with which they underwent cleavage and formation of a blastocoel. Blastocyst-stage embryos (i.e. those having a blastocoel) were transferred using the drawn Pasteur pipet (described above) in groups of 20 or less to 0.25 ml of Eagles' medium modified according to Spindle and Pedersen (91). The medium was contained in each well of an 8-chambered tissue-culture slide. Morulae (embryos that have undergone cleavage and compaction but which do not have a blastocoel) were transferred to separate wells. The number of blastocysts, morulae and dead or degenerating embryos was noted. Morulae and blastocysts were counted with a dissecting microscope using about a 50X magnification. Discrepancies in the number recovered and the number transferred were noted but typically 99% of all embryos could be accounted for. The embryos were returned to the incubator for an additional four days of incubation under the conditions described earlier.

After four days of additional incubation the slides containing the embryos were removed from the incubator, the medium poured gently off and the plastic wells removed from the underlying gasket. The frequency with which the blastocysts and morulae formed trophectoderm outgrowths and the frequency with which the trophectoderm outgrowth had a proliferated inner cell mass was noted. These analyses were done with the aid of a dissection microscope typically operated at 60-80X magnification. After counting the trophectoderm outgrowths and the inner cell masses, the slides were fixed and stained with gentian violet in 70% ethanol.

Thus for any assay the following reproductive parameters were recorded:

(1) The number of successful matings;
(2) The number of 2-cell stage embryos and the number of unfertilized or degenerating ova in each female;
(3) The number of 2-cell stage embryos transferred into modified Ova Culture medium;
(4) The number of blastocysts and morulae developing from the 2-cell stage embryos;

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(5) The number of trophectoderm outgrowths developing from the blastocysts and morulae and therefore from the 2-cell stage;

(6) The number of inner cell masses at the trophectoderm outgrowth stage and therefore the number of inner cell masses developing from blastocysts, morulae and ultimately from the 2-cell stage.

2.2.5 Cytogenetic Assays.

2.2.5.1 Sperm head abnormalities. The criteria of Wyrobek and Bruce (92) were applied to spermatozoa rinsed from the cauda and caput epididymes. Males were selected randomly and killed by cervical dislocation. The testes were excised and the caput and cauda epididymes were dissected free of fat and then minced in phosphate buffer (1 ml) in a watch glass. A drop of the sperm suspension was placed on a clean microscope slide and spread evenly. The slide was fixed by passing through a flame and the spermatozoa were stained with crystal violet. The spermatozoa were viewed with a 40X planar objective and 200 were scored as normal or abnormal. The abnormals were further classified into headless, two-tailed, hookless, two headed, rounded or other. Details of the preparation and assay are found in Wyrobek and Bruce (93).

2.2.5.2 Translocations in spermatocytes. The method of Evans et al (94 was used. The testis was removed and the tunica torn with forceps. A suspension of the seminiferous tubules was made and spermatocytes prepared by dropping a distance of approximately one meter onto a clean microscope slide. The slide was fixed and stained and the resulting preparation assayed for the presence of quardrivalents in either the ring or chain configuration. Details of the preparation are found in Evans (94).

2.2.5.3 Micronuclei in embryos. The technique described by Muller (95) was used. Blastocyst stage embryos were placed in hypotonic buffer and then placed in one well of a three well spot plate. They were allowed to swell for 6-15 min and then transferred to another well containing alcohol-acid fixative (1:3 glacial acetic:ethanol). They were immediately removed from the fixative and placed in a small drop of the fixative on a clean microscope slide. Air was blown onto the embryo to flatten and spread the blastomeres. After drying for three days they were stained with either Giemsa or ethidium bromide. Ethidium-bromide stained embryos were viewed under the florescent microscope and the number of micronuclei per embryo was determined. The criteria of Muller et al (95) were applied to the identification and classification of micronuclei and complete details of the procedure can be found therein (95).

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2.3 DATA ANALYSIS

2.3.1 Dominant lethal mutant rates.

The following relationship was used to describe the dominant lethal mutant rates (DLM) for various developmental stages for all the experiments:

$$DLM = \{1 - (a/a')\} \times 100$$

where a and a' are the mean rate of successful development from one developmental stage to another in experimental (a) and control (a') groups respectively. DLM was further defined as DLM_p (preimplantation) for development from the 2-cell stage to the blastocyst stage; DLM_i (implantation) for development from the blastocyst stage to the trophectoderm outgrowth stage; DLM_e (early postimplantation for development of a proliferated inner cell mass on the trophectoderm outgrowth; and DLM_o (overall) for development from the 2 cell stage to the trophectoderm outgrowth stage with proliferated inner cell mass.

Data for individual matings were collapsed to time post treatment intervals that corresponded to the periods necessary for treated spermatogenic stages to complete spermatogenesis. Thus matings on the day of treatment and 3 and 7 days post treatment were combined into a single data point for treated spermatozoa. Similarly, matings between 8 and 21 days post treatment were combined for treated spermatids. Matings between 22 and 35 days were combined into treated spermatocytes and matings more than 35 days post treatment were combined into treated spermatogonia. These increments are the ones described by Oakberg (21) for spermatogenesis in the mouse but the timing was not verified for the strain used here.

In experiments involving x-irradiated or argon-irradiated males there were typically 4 to 6 data points for each dose for each mating. For neutron-irradiated males and for experiments involving treated females the offspring from each mating were not pooled with those from other matings and therefore each data point was derived from 25 to 40 matings. For both methods of data collection the results were analyzed for significance at the 5% level by both parametric (t-test) and non-parametric (Mann-Whitney U-test) methods.

The data for the dominant lethal mutant rates were plotted as a function of

dose using linear-linear coordinates. Data in the dose modification studies are presented in a Table.

2.3.2 Cytogenetic Studies.

The mean number of blastomeres in an embryo at a given time after mating was determined and the data collected in a table. Establishing criteria for micronucleus formation presented some problems in interpretation and we therefore relied on the definition for micronuclei presented by Muller et al (95) and determined the rate in blind samplings. Because of this uncertainty complete data sets were not accumulated and this analysis must still be regarded as preliminary.

SECTION 3
RESULTS

3.1 EFFECTS OF ACCELERATED ARGON NUCLEI IN MALE GERM CELLS

3.1.1 Cell killing and dominant lethal mutation induction in spermatozoa derived from irradiated spermatozoa, spermatids, and spermatocytes.

The mice were irradiated with 0.5, 1.0, 1.5, 2.0 or 2.5 Gy.

Since it takes 35 days for murine spermatogonia to complete spermotogenesis and reach the epididymis, matings that take place within the first five weeks after treatment assess damage in spermatozoa treated in the spermatozoal, spermatid or spermatocyte stages.

Analysis of mutation induction in these stages was confounded by technical problems in the assay. In general these problems involved contamination by either mold or bacteria in one or more dishes from one or more test doses. An unusually low level of successful development in controls suggested that the medium was compromised in all samples to some extent but that the growth of the contaminants was checked by the antibiotics contained therein. Repeated efforts to find the source of the contamination during the initial testing period failed to give a definitive answer. The problem abated soon thereafter and we still have no precise idea as to the source of the contamination. For this reason, data accumulated in this period were handled in a different manner than usual. Rather than assay the rate of successful development in each dish of embryos, all the data for each dose point were collapsed over the respective cell stage. Although the survival to each endpoint represents the effect due to radiation and a larger-than-normal contribution due to the challenge of in vitro growth, the populations investigated are probably large enough so that the data are representative of the radiation effects themselves.

Irradiation of spermatozoa and spermatids by accelerated argon ions gave results that were similar in many respects. The fertilization rate was unaffected indicating that spermatozoa were produced that could undergo all the necessary

reactions for fertilization (motility, capacitation, acrosomal penetration, etc.). In other studies we found that failure to fertilize is usually associated with reductions in the sperm-count to 10% or less of control levels. Because the fertilization rate for these irradiated stages was unaffected, the data indicate that more than 10% of the spermatozoa from irradiated precursor stages were available for fertilization.

Because the fertilization rate was unaffected, the data suggest that any reduction in litter size or reproductive integrity found in vivo would be due to the introduction of a compromised male pronucleus in the ova. The embryos derived from irradiated spermatozoa and spermatids demonstrated reduced developmental capacity relative to controls and therefore these embryos expressed induced dominant lethal mutations. The mutagenic lesions were expressed throughout development in a dose-dependant manner (Table 1). When the overall dominant lethal mutant rate was plotted as a function of dose, the final rates for spermatozoa derived from treated spermatozoa and spermatids were essentially the same although the rate at lower doses was higher in spermatids (fig 1). At still higher doses one would anticipate that the slope would decrease as lethal lesions are wasted in cells that already are doomed to die.

Table 1. Frequency of successful development and dominant lehality in embryos sired by Argon-irradiated males.

	DNA, Gy	2-cell	2→B[1]	B→TBOG	TBOG→ICM	2→ICM
SPERMATOZOA	0	318	.67	.76	.52	.35
	0.5	273	.61 (.09)	.72 (.05)	.67 (-.29)	.29 (.17)
	1.0	279	.60 (.11)	.46 (.39)	.57 (-.10)	.27 (.23)
	1.5	524	.57 (.15)	.39 (.49)	.53 (-.02)	.21 (.40)
	2.0	274	.49 (.27)	.66 (.13)	.58 (-.12)	.19 (.46)
	2.5	236	.44 (.34)	.56 (.26)	.48 (.08)	.12 (.66)
SPERMATIDS	0	2310	.78	.73	.53	.30
	0.5	1777	.62 (.21)	.77 (.05)	.41 (.23)	.20 (.33)
	1.0	2001	.45 (.42)	.29 (.62)	.35 (.34)	.04 (.87)
	1.5	1132	.29 (.63)	.51 (.34)	.47 (.11)	.08 (.73)
	2.0	2188	.39 (.50)	.48 (.38)	.38 (.28)	.07 (.77)
	2.5	2297	.29 (.63)	.42 (/45)	.36 (.32)	.04 (.87)
SPERMATOCYTES	0	1100	.86	.81	.66	.43
	0.5	826	.88 (-.02)	.74 (.09)	.51 (.23)	.33 (.23)
	1.0	262	.63 (.27)	.87 (-.07)	.51 (.23)	.27 (.37)
	1.5	76	.61 (.29)	.54 (.33)	.40 (.39)	.13 (.70)
	2.0	50	.32 (.63)	.75 (.07)	.58 (.12)	.14 (.67)
	2.5	58	.40 (.53)	.61 (.25)	.21 (.68)	.05 (.12)
SPERMATOGONIA	0	3797	.84	.82	.75	.52
	0.5	3155	.87 (-.04)	.83 (-.01)	.68 (.09)	.50 (.04)
	1.0	3652	.89 (-.06)	.82 (0)	.59 (.21)	.43 (.17)
	1.5	2757	.89 (-.06)	.82 (0)	.65 (.13)	.47 (.10)
	2.0	3566	.86 (-.02)	.80 (.02)	.63 (.16)	.44 (.15)
	2.5	2580	.87 (-.04)	.79 (.04)	.60 (.13)	.42 (.19)

[1] frequency of successful development; ( ), Dominant lethal rate.

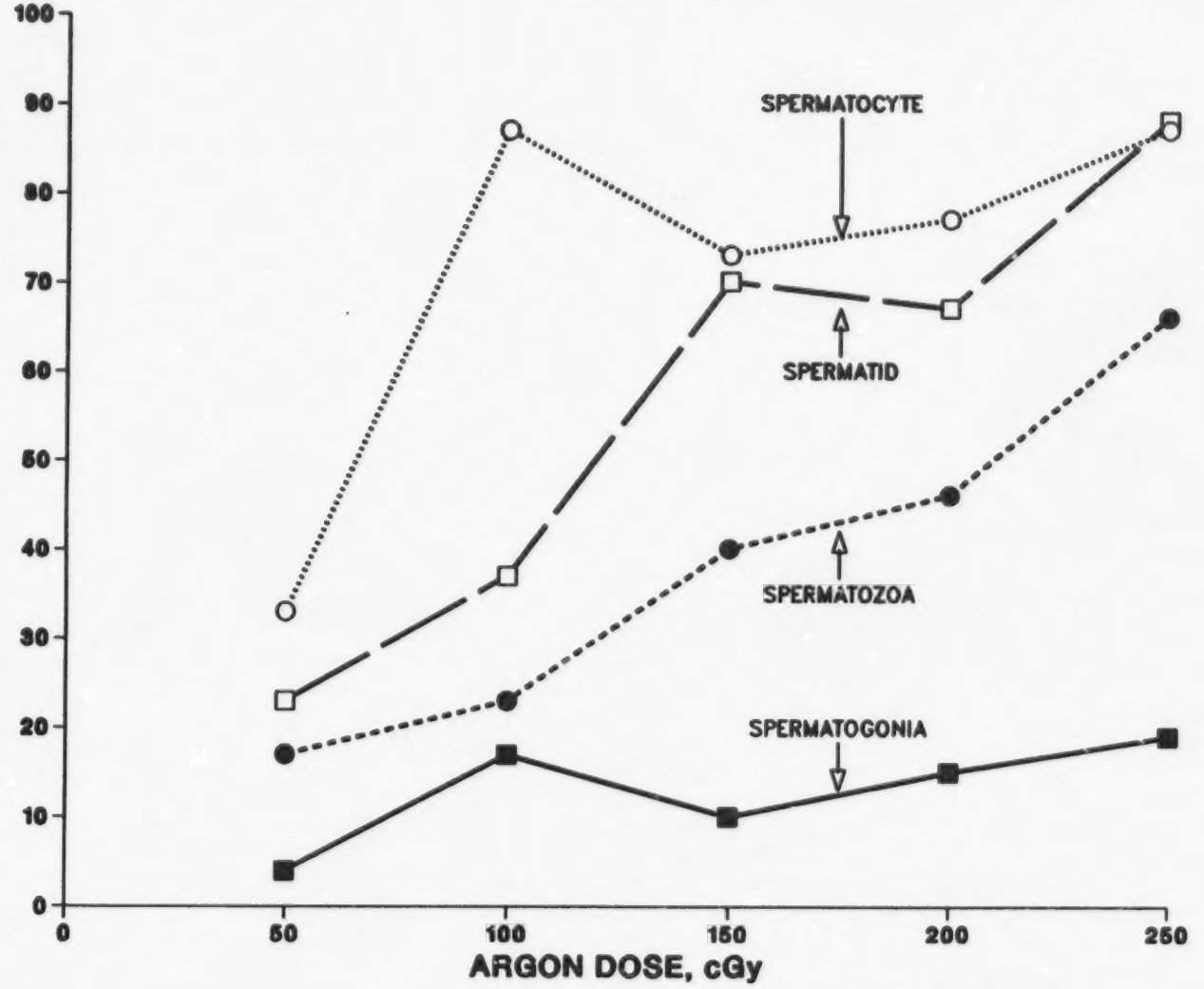

Figure 1. Dominant lethal mutation induction in male germ cells by 95 keV/μm accelerated argon nuclei.

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The fertilization rate was depressed in spermatozoa derived from argon-irradiated spermatocytes. Other work (not shown) indicates that this effect is due to oligo- and azo-spermia due to the treatment. The reduction in the fertilization rate was dose dependent with respect to both its extent and duration. Because of this, the analysis of radiation induced dominant lethal mutations (Table 1) is valid only for low dose groups where the fertilization rate remains reasonable. When plotted as a function of dose, the dominant lethal mutant rate in spermatozoa derived from treated spermatocytes is linear and has a somewhat higher initial slope than the rates for irradiated spermatozoa and spermatids (Fig 1).

3.1.2 Cell killing, mutagenesis and clastogenesis in spermatogonia.

Fertility returned to all groups after about 100 days and this was taken as the time when cells irradiated as stem cell spermatogonia were found in the ejaculate. Matings from 35 to 100 days post treatment were probably representative of cells irradiated as differentiating spermatogonia. No reduction in the fertilization rate was detected in animals mated 100 or more days after treatment indicating that the sperm-count had returned to normal or near normal levels and that the spermatozoa derived from the irradiated precursor were not compromised with respect to their ability to reach the egg, penetrate the membrane and inject the pronucleus. The offspring fertilized by spermatozoa derived from treated spermatogonia were developmentally impaired (Table 1) indicating the presence of induced dominant lethal mutations.

The overall dominant lethal mutant rate is described by a linear function of dose (fig 1). There was no evidence of a threshold and the embryos failed at all times during development (Table 1). Analysis of these data with either parametric or non-parametric tests demonstrated highly significant differences ($P < .005$) and, when considered individually, significant differences for the 2.0 and 2.5 Gy exposures ($P < .05$).

Cytogenetic analysis of spermatozoa in the cauda epididymis 8 or more weeks after radiation revealed no significant decrease in sperm counts (Table 2). This result agrees with the indirect analysis of sperm counts as assessed in the fertilization assay but whereas that assay could only state that killing was less than 90%, the sperm counts indicated no decrease in the number of spermatozoa. The cytogenetic assay does not address the fidelity or viability of the spermatozoa.

Table 2. Cytogenetic analysis of spermatozoa and embryos derived from Argon-irradiated spermatogonia.

Dose, Gy	# embryos	blastomeres/ embryo	mn/embryo	sperm cts ($x10^{-6}$ ml^{-1})	testis wt (mgms)
0	51	47.6	1.82	27.0 $\pm$ 4.6	148 $\pm$ 21
0.5	46	42.5	2.09	26.0 $\pm$ 9.3	129 $\pm$ 25
1.0	39	58.1(1)	5.51*	27.5 $\pm$ 5.5	129 $\pm$ 12
1.5	21	49.4	3.04*	28.0 $\pm$ 8.2	120 $\pm$ 24
2.0	22	44.3	3.27*	24.9 $\pm$ 4.9	120 $\pm$ 13
2.5	20	49.4	1.15	23.7 $\pm$ 6.9	109 $\pm$ 20*

* $P < .05$, t-test

(1) may include degenerating or incomplete cells.

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When testis weights were measured, only the 2.5 Gy group showed a significant decrease. However, the weight loss did not correlate with sperm counts and may therefore affect an aspect of the testis not related to germ cell production. When embryos fertilized by spermatozoa derived from irradiated spermatogonia were analyzed, an increase in the mean number of blastomeres and the micronuclei per embryo was found in the group receiving 1.0 Gy. This group behaved anomalously in the dominant lethal assay for post meiotic cell stages and these observations may be correlated. However, no other group demonstrated deviations from the control levels and therefore these measurements appear inadequate to estimate the genetic risk.

3.2 EFFECTS OF FISSION SPECTRUM NEUTRONS IN MALE GERM CELLS

The mice were irradiated with 0.25, 0.5, 1.0, 1.5 or 2.0 Gy.

Because of the difficulties encountered when the embryos were pooled into groups of 150, a new procedure was initiated in which the offspring from each mating were incubated in a dish rather than combining the litters from several mice together. In addition the mice were given several mating experiences before being irradiated. This served two purposes: it allowed us to remove mice with inherent reproductive deficiencies from the population and it gave the staff the practice necessary for handling the much increased technical aspects. A suitable mechanism was established whereby the embryos from a given male were identified and set up in culture without confusion. Once this was established the males could be assigned randomly to a dose group and then maintained by code only. Thus we were able to vastly improve our statistical power and at the same time reduce inherent bias in the analysis.

Immediately after irradiation the mice were returned to our animal care facility and the dominant lethal analysis was initiated.

3.2.1 Cell killing and dominant lethal mutation induction in spermatozoa derived from irradiated spermatozoa, spermatids and spermatocytes.

The fertilization rate was unaffected by the treatment for the first three weeks. However, the developmental capacity of the embryos in vitro was dramatically reduced. Embryo failure occurred at all stages of development with no readily apparent pattern (Table 3). A plot of the overall dominant lethal mutant rate as a function of dose reveals a linear relationship for both irradiated spermatozoa and spermatozoa derived from irradiated spermatids (fig 2). Spermatids were about twice as sensitive.

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Table 3. Dominant Lethality Induced by Fission Spectrum Neutrons.

	Dose, Gy	DLM_p	DLM_l	DLM_e	DLM_o
SPERMATOZOA	0.25	-3.7	3.4	2.6	2.7
	0.50	7.8	5.0	-0.4	14.0
	1.00	7.4	11.9	5.9	31.1
	1.50	23.2	24.2	2.4	39.4
	2.00	37.1	23.2	-2.4	54.6
SPERMATID	0.25	13.6	17.2	3.4	25.0
	0.50	22.9	16.7	3.1	32.0
	1.00	30.5	31.9	10.4	56.4
	1.50	49.3	41.5	13.3	71.2
	2.00	56.8	46.7	10.5	78.8
SPERMATOGONIA	0.25	-0.8	-5.4	4.0	-5.0
	0.50	0	-3.1	3.0	0
	1.00	-0.2	-2.3	2.6	0
	1.50	2.2	-4.0	1.3	6.4
	2.00	6.7	5.6	5.2	16.0

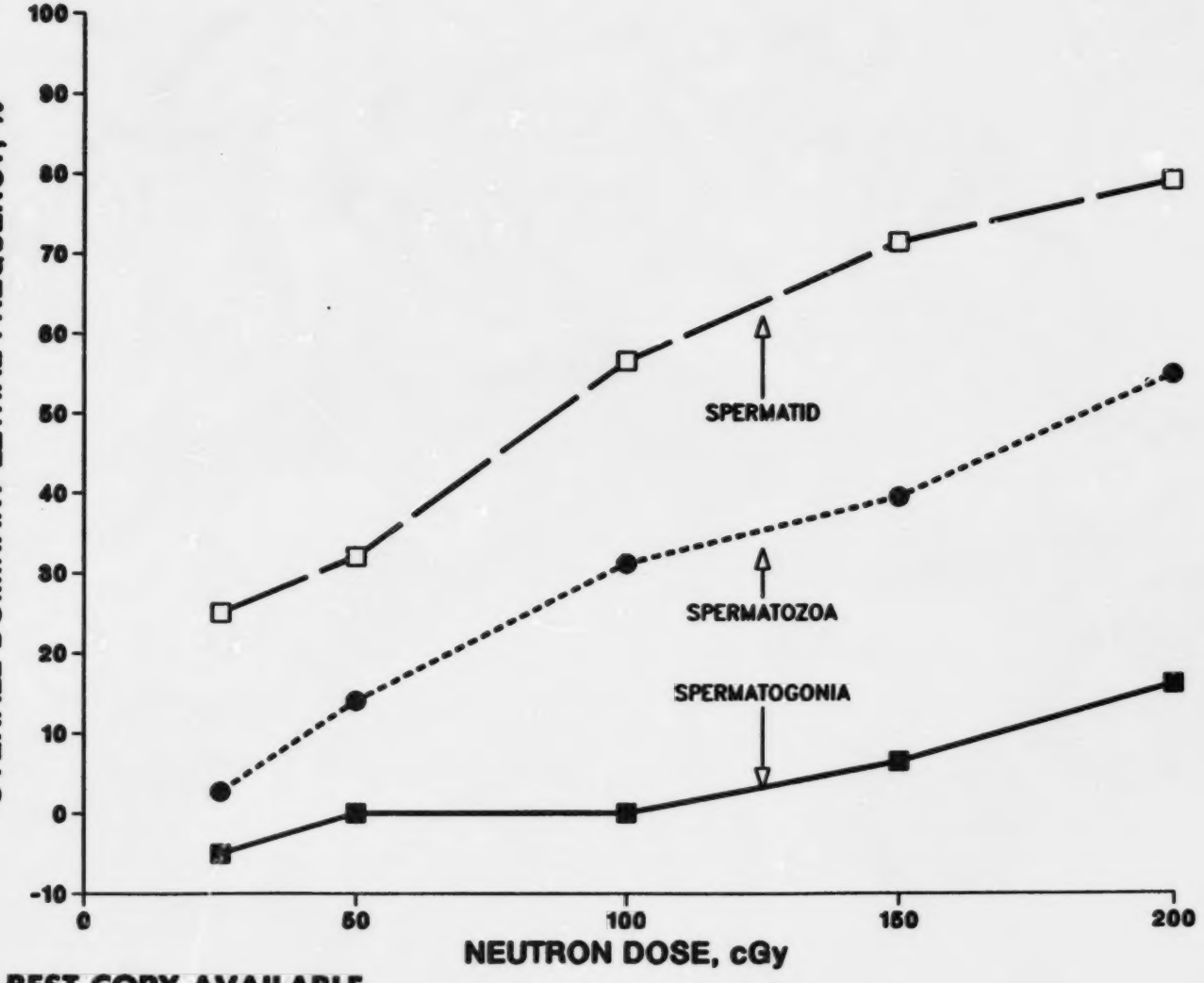

Figure 2. Dominant lethal mutation induction in male germ cells by fission spectrum neutrons.

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The data for irradiated spermatocytes was influenced by radiation-induced sterility most likely reflecting cell killing. However sufficient numbers of fertilizations occurred to generate complete dose-response curves for the overall dominant lethal mutant rate (fig 2). Spermatocytes were more sensitive than spermatids but the curve gave indications of reduced efficiency at high dose suggesting that lethal mutations were being wasted in cell already containing a lethal event. The transient sterility was dose-dependent with respect to its extent and duration.

3.2.3 Cell killing and dominant lethal mutation induction in spermatozoa derived from spermatogonial stem cells.

All the groups returned to full fertility at about 100 days and this time post treatment was established as the cut-off for analyses of spermatozoa irradiated as spermatogonial stem cells

When the overall dominant lethal mutant rate was plotted as a function of the dose to irradiated spermatogonia, the embryos sired by males receiving 0.25, 0.5, or 1.0 Gy had a negative or zero mutant rate (i.e. they developed as well or better than the embryos sired by unirradiated controls). These results may be interpreted in two ways - either the radiation preferentially killed spontaneously mutated germ cells in the population or the control population had an unusually high background mutation rate. In my experience with this assay this is the only time that embryos derived from mutagenized males performed better than their untreated counterparts and I have chosen to analyze the data in terms of the second argument (that the control population had an unusually low rate of successful embryogenesis in vitro despite efforts to make the groups as homogenous as possible at the beginning of the study). For setting risk limits this is the only prudent approach since the only study that purports to demonstrate a "radiation adaptive response" does so only at much lower doses (see discussion, section 5). Using this bias, the data indicate that dominant lethal mutants are detected more frequently with increasing dose and that the kinetics of induction is consistent with a simple linear rate (fig 2).

Both parametric and non-parametric tests indicate that there is significant overall mutation induction in male mice irradiated with fission spectrum neutrons ($p < .01$). Spermatogonia receiving 2.0 Gy had significantly higher mutant rates than did spermatogonia receiving 1.0 or less Gy. Spermatogonia receiving 1.5 Gy

were not different from those receiving more or less radiation. These results suggest that using this type of experimental design the in vitro dominant lethal mutant assay can detect a mutant rate of between 6.4% and 16% with a 95% probability.

3.3 MUTATION INDUCTION IN OOCYTES BY X-RADIATION.

A group of 100 untreated males of proven mating ability were maintained for pairing with irradiated and control females. For each experiment the males were assigned randomly to a given treatment group so that the results would not be biased by the male contribution to embryo viability.

X-irradiation was performed 2h before hCG injection and pairing which corresponds to about 16h before fertilization. The embryos from each mating were incubated in separate dishes and analyzed for successful development in vitro.

A dose-dependent increase in dominant lethal mutants was detected (Table 4, fig 3). Embryo lethality was expressed at each developmental endpoint. The induction curve appeared to be biphasic with an initial steep region followed by a less steep final slope. Because the oocyte is irradiated in a non-dividing stage one can apply Poisson statistics to give the dominant lethal mutation rate. This is seen to be a linear function of dose suggesting that the shape of the mutation induction curve is influenced by the simultaneous induction of more than one lethal lesion (fig 3). These data also suggest that for the oocyte, the dominant lethal mutation rate should be used to set exposure limits.

Table 4. Preimplantation, implantation, early post-implantation and overall dominant lethal frequencies (dlf) expressed by embryos derived from irradiated oocytes [1].

Dose, Gy	Number of 2 Cell Embryos (n, r)[2]	DLFp	DLFi	DLFe	DLFo
0	1409 (77,7)	–	–	–	–
0.5	694 (32,2)	.07 -.10	.15	.14	
1.0	599 (37,3)	.03	.12	.18	.32
1.5	929 (60,4)	.07	.12	.14	.28
2.0	513 (27,2)	0	.30	.09	.41
2.5	557 (32,2)	.19	.22	.17	.37
3.0	844 (60,4)	.15	.15	.19	.43
3.5	430 (31,2)	.23	.24	.21	.50
4.0	452 (31,2)	.42	.20	.23	.80
4.5	956 (53,4)	.38	.40	.21	.63

(1) See text for derivations and method of calculation.

(2) n, number of females; r, number of trials.

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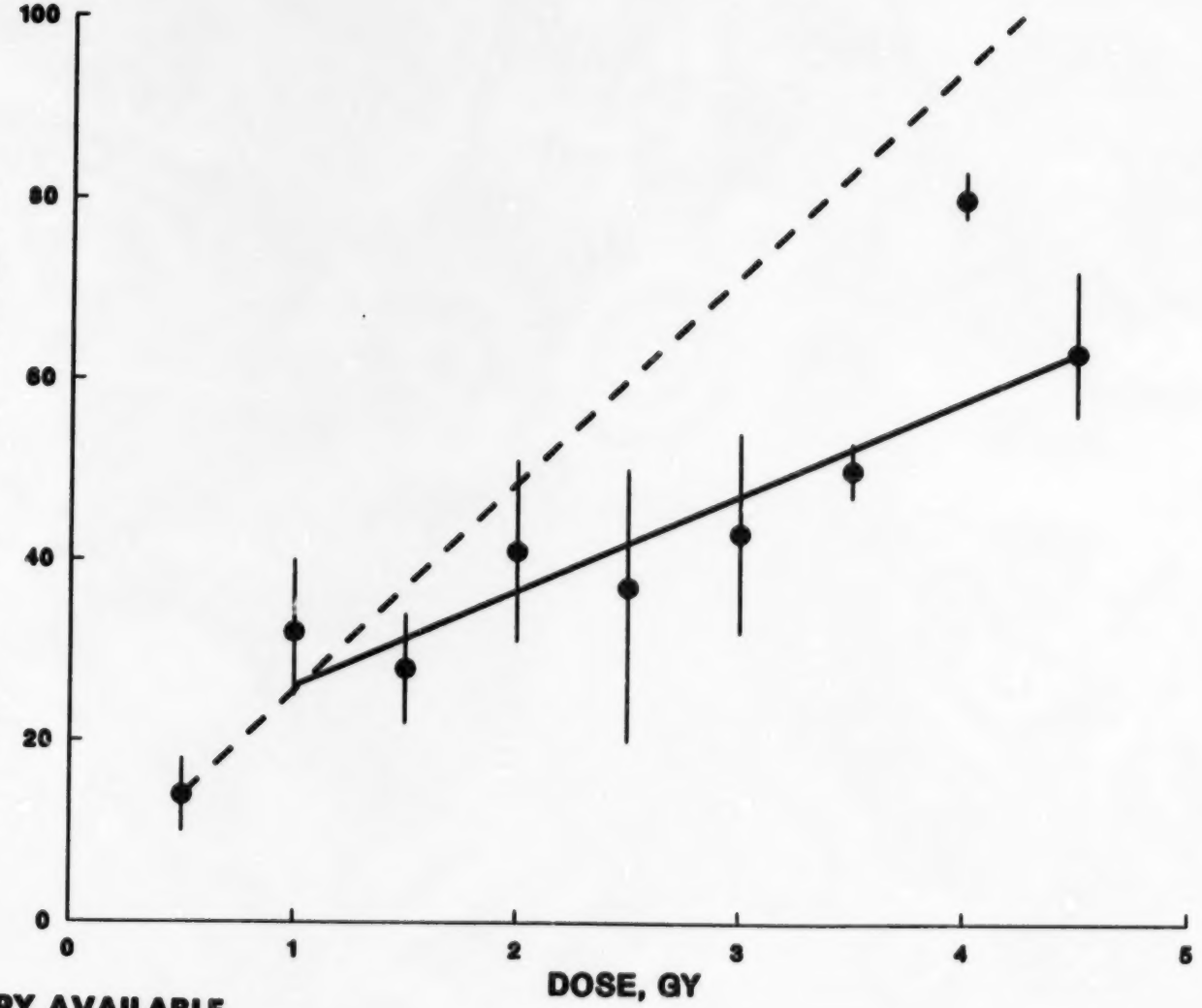

Figure 3. Dominant lethal mutation induction by x-radiation in preovulatory oocytes.

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Females that were irradiated but which did not mate were kept for another two weeks and induced to ovulate again. Using this protocol oocytes that were irradiated while in the sensitive non-follicular stages could be evaluated for dominant lethal mutations. However, even those exposed to the lowest dose of radiation (25 cGy) showed substantial killing and therefore this part of the assay was discontinued.

The mutation induction curve for irradiated murine oocytes is substantially different from data in the literature that show the dose-response kinetics for either in vivo dominant lethality or for chromosome aberrations in this system (64,69-73).

3.4 DOSE MODIFICATION OF MUTAGENESIS.

In preliminary experiments (not shown), data suggested that pretreatment of a stem cell population with a combination of the chemotherapeutic drugs mechlorethamine and procarbazine modified the subsequent response to the lethal effects of ionizing radiation. To test this in germ cells, male mice were treated with the drugs and then irradiated one week later. Five weeks after the irradiation the mice were enrolled in the dominant lethal study. Mice receiving the drugs alone or X-radiation alone served as the basis of comparison.

Only four mating intervals were obtained after the mice returned to full fertility and before the onset of a seasonal influence in embryo growth and reproductive fitness. Though limited, the data suggest that pretreatment of the spermatogonial stem cell with chemotherapeutic drugs reduces the subsequent rate of radiation-induced mutagenesis (Table 5). Radiation alone caused about a 12.4% rate of mutagenesis, but pretreatment with the drugs alone reduced this substantially. However, these data are complicated by the fact that the drugs alone did not cause significant mutagenesis. At least one of these regimens (MP) was subsequently found to be mutagenic in the absence of radiation. Other work which included these 2 drugs and vincristine showed that the overall mutation rate due to the 3-drug routine followed by 2.0 Gy x-radiation was subadditive with respect to the two modalities given independently.

Table 5. Dose modification by pretreatment with mechlorethamine, procarbazine or both on x-ray induced dominant lethality.

	Number of Embryos Assayed				Dominant Lethality			
tmt	trans	blast	tbog	icm	DLM_p (3)	DLM_l	DLM_e	DLM_o
Control	1902	1448	1257	999	–	–	–	–
X	2729	1956	1523	1255	5.8	10.3	-3.7	12.4*
PM	2578	2280	1955	1477	0	1.2	4.9	-9.0*
PMX	2081	1584	1438	1185	0	-4.6	-3.6	-8.4*
MP	2482	2178	1845	1349	-15.0	2.4	8.0	-3.5
MPX	1576	1311	1038	846	-9.0	8.8	-2.5	-2.2

(1) 2.0 Gy. P = procarbazine, M = mechlorethamine. Order of letters indicates order of treatment.

(2) trans = 2 cell stage transferred; blast = blastocysts; tbog = trophectoderm outgrowth; icm = inner cell mass at trophectoderm outgrowth stage.

(3) DLM = Dominant lethality expressed before blastocyst formation (DLM_p), between blastocyst formation and trophectoderm outgrowth (DLM_l), for inner cell mass formation at tbog (DLM_e), at anytime during in vitro development (DLM_o).

* $P < .05$, t - test

SECTION 4
DISCUSSION

4.1 RADIATION-INDUCED MUTATIONS IN MALE GERM CELLS.

4.1.1 General.

The in vitro dominant lethal assay detected mutations induced by densely ionizing radiation in pre-meiotic, meiotic and poet meiotic male germ cells. The highest rates are detected in the spermatocyte, but the analysis is complicated by radiation induced cell killing. The spermatids are the next most sensitive followed by the spermatozoa. Although the rates detected in spermatogonia are less than the rates in the meiotic and post-meiotic stages, this cell deserves special consideration since mutations in it represent the long-term mutagenic hazard to the irradiated male.

The rate of mutation induction plotted as a function of dose can be adequately described by a straight line. This implies that the mechanism of damage induction is by single hits within the sensitive target. Both fission spectrum neutrons and 95 keV/μm particles induce dominant lethal mutations in spermatogonia with about the same kinetics.

The doses tested induced a transient sterility which was overcome within about one hundred days post irradiation. Sperm counts returned to near control levels and were well above the number necessary for full fertililty.

Manifestations of genetic damage at the chromosomal level were more difficult to demonstrate and quantify. There was evidence of micronucleus formation in the embryos but the signal to noise ratio was too high to make definitive statements about its reliability as a predictor of radiation damage.

Sperm head abnormalities were detected but at rates that did not correspond to the levels of dominant lethality detected by the in vitro protocol.

Dominant lethal testing appears to be the preferred method for detecting radiation-induced damage to the germ cell. While testing in post meiotic stages is valuable for establishing that the germ cell was indeed exposed, the long term genetic hazard is best estimated in studies of the effects in irradiated spermatogonia.

The in vitro methodology appears to be more sensitive and cost effective than the in vivo approach.

4.1.2 Exposure to accelerated charged particles.

The data indicate that damage induced in non-dividing germ cells and expressed in the embryo is manifest as both a failure to successfully develop to the trophectoderm outgrowth stage and then to successfully differentiate and proliferate an inner cell mass. In contrast, the damage induced in the dividing germ cell is manifest only as an inability to differentiate and proliferate an inner cell mass. It seems likely that damage leading to embryo failure is culled from the population of spermatogonia, either by repair or by elimination of the cell in which it is found. A diminution in detectable chromosome damage at meiosis has been reported in mice (55) and is marked contrast to the high rate of damage that successfully gets through meiosis in humans (6,13). Surely chromosomal damage is induced since it is expressed in embryos fertilized by post meiotic cells. It is unlikely that the location of the spermatogonial stem cell within the seminiferous epithelium prevented damage from being induced.

There appears to be a component of damage that causes genetic effects within the embryo that is not detectable by cytogenetic methods. The nature of embryo death seen when fertilization is by a spermatozoa carrying damage induced by densely-ionizing charged particle radiation is similar to that seen in genetically defined matings with trisomic or nullisomic mice (96) suggesting that the nature of the lesion is the same for both conditions. A working hypothesis at this stage is that the kind of damage detected represents a form of structural aberration involving a part of a chromosome that is not readily detected by karyotyping. Lethality is due to a gene dosage effect with the accompanying hypo- or hyper zygosity.

The shape of the dose response curve suggests that the rate at which damaged spermatogonia contribute to the pool of fertilizing spermatozoa is not changed appreciably over the course of the experiment. In other words, the damaged spermatogonium is neither selected for nor against during spermatogenesis. Clearly the nature of the defect is not lethal to the spermatogonium per se since it is transmitted for a considerable time after exposure. The data do not discern a change in homeostasis within the testis since the average sperm-counts have a wide variance even in untreated animals. In the absence of data to the contrary I

conclude that the damage detected within the embryo is permanently encoded in the germ line but this damage in no way adversely effects the ability of the male to mate. Therefore such damage will be transmitted to the population by the irradiated individual and by those offspring where the same or similar damage is not lethal. Damage to densely ionizing charged particles therefore has the potential to permanently alter the gene pool.

In other experiments, embryo failure at blastocyst formation or trophectoderm outgrowth when fertilization was by spermatozoa derived from irradiated spermatogonia was detected only when the ionization density of the particle was about 150 keV/μm (Goldstein, unpublished). Particles with this radiation density also demonstrate a reverse fractionation effect for cell survival (i.e. fewer cells survive when a given dose is delivered in several fractions than when the same dose is delivered in a single exposure, ref. 78). This may be due to cell-cycle dependent actions in the dividing spermatogonia and such effects are not found at lower ionization densities. Since the nature of the stem cell spermatogonium remains elusive, it is difficult to say whether strategies to reduce damage should include consideration of cell cycle. Based on the current experimentation, there is no compelling reason to dismiss attempts to modify the mutation yield by agents that act in either dividing or non-dividing cells.

The studies performed with accelerated argon nuclei indicate that genetic damage manifest as a dominant lethal mutation is induced with high efficiency and therefore exposure to this particle or related radiations poses a genetic hazard.

4.1.3 Exposure to fission-spectrum neutrons.

The sensitivity of the stages of cells in the germinal epithelium to fission spectrum neutrons is: spermatids > spermatozoa > spermatocytes >> spermatogonia. For the post meiotic and meiotic stages, the induction of dominant lethal mutations could be adequately described as a linear function of dose. This linearity appeared to extend to the irradiated spermatogonial stem cell also although the data do not permit an unequivocal statement about this aspect. Certainly for risk assessment, the most prudent approach is to consider that mutations are induced in spermatogonia as a linear function of dose.

The mutagenesis detected is due to neutrons alone because the source permitted filtering the concomitant gamma radiation. The data demonstrate that the fission spectrum neutron does cause permanent heritable damage in the germ cell.

When meiotic or post-meiotic cells are exposed to fission spectrum neutrons, damage that causes cellular reproductive death (failure to form a blastocyst) as well as damage that interferes with differentiation (failure to form a trophectoderm outgrowth or proliferate an inner cell mass) is induced. Since cells in division (spermatocytes) have reduced survival, the data suggest that a considerable amount of the type of damage that causes cellular reproductive death is removed during cell division. Repair of such damage as an alternative mechanism seems unlikely since the repair potential of the post-meiotic cells is much less than their premeiotic counterparts. The resulting sterility is dose dependent with respect to duration and extent but is overcome within about 100 days after 2.0 Gy.

Permanent damage is detected in embryos sired by spermatozoa derived from irradiated spermatogonia. This damage is manifest only as a failure to differentiate and proliferate an inner cell mass atop the trophectoderm outgrowth. The nature of the damage is unproven but deletion resulting in aneuploidy is a likely candidate. The nature of the mutation induction curve indicates that only single hit events are needed and therefore the contribution of two -hit events (e.g reciprocal translocations) is unlikely unless a single ionization causes 2 discrete lesions in separate chromosomes. While this appears unlikely, such a possibility cannot be excluded.

Data analysis of the distribution of mutagenesis within the population is still incomplete but initial indications are that the population is not homogenous with respect to its sensitivity. For example, a male (#186) that received 0.5 Gy of fission spectrum neutrons did not undergo radiation-induced sterility to the extent that other similarly irradiated males did. His dominant lethal mutant rate also appeared to be lower than that of his cohorts. Attempts to breed this male and recover a "resistance" trait were unsuccessful because the male died soon after they were initiated. The apparent cause of death was lymphoma, although this will require histological verification since the symptoms for lymphoma and lymph-node infection are the same. It is interesting to note that a deletions leading to predisposition to cancer has been identified in humans (97,98). There is no reason

that humans should not demonstrate a range of genetically-determined sensitivities to radiation and therefore the mouse model alluded to may be particularly relevant. A corollary to this is that setting exposure standards should at least consider the reaction of the most sensitive individuals in the population at risk.

The shape of the mutation induction curve suggests that little will be gained if the dose of neutrons is delivered in many small fractions or at low dose rates. A reduction in the rate of mutagenesis by accelerated neutrons by treatment with the sulphydryl-containing compound WR-2721 (or its active component, WR 1065) has been reported by Sigestad et al (99). This suggests that at least some of the damage induced by fission spectrum neutrons may be prevented (or its repair augmented) by sulfhydrl-containing compounds. Since the LET spectrum for fission spectrum neutrons is very broad, such modification may be acting only on damage induced by the sparsely-ionizing components. Alternatively, since gamma radiation was not excluded in the other studies, it may be that neutron-induced damage cannot be prevented or modified by chemical intervention. This question will have to be investigated in studies directed to this point.

4.1.4 Comparing mutagenesis by accelerated charged particles fission spectrum neutrons.

Fission spectrum neutrons and accelerated charged particles with ionization density of about 95 keV/μm induce genomic damage in the male germ cell in manners that are very similar, both on a quantitative and qualitative basis. An underlying hypothesis to these studies was that both particles should be at, or near the optimal efficiency for mutation induction. Their similarities would tend to support this assumption although it is possible, indeed likely, that other, more efficient sources of radiation will be described. The doses necessary to demonstrate significant levels of mutation induction are far below the whole-body lethal threshold and therefore the hazard from exposure to either of these sources is both real and quantifiable.

Exposure to either of these forms of radiation can come from a variety of environmental and industrial settings associated with the Department of Defense. These range from exposure to cosmic rays while in high altitude aircraft or satellites, to workers near reactors either in weapons manufacture or nuclear-powered vessels, as well as casual exposures in mining and even housing in concrete

reinforced enclosures. The data in the model system investigated here suggest that men exposed to any level of radiation of this type (i.e. that having a component of densely ionizing radiation) are subject to some risk to their reproductive integrity. As was mentioned in the introduction however, this risk must be considered in relation to other risks involved in the performance of one's normal functions. The data do not support a threshold concept and therefore it is the responsibility of the individual to avoid unintended exposure to such sources. This should be easily accomplished within the structure of safety-related recommendations within each job classification.

That the level of induced dominant lethal mutation at the highest exposures approaches the "spontaneous" rate of abortion in the population as a whole should not be a source of comfort. It must be emphasized that the genetic endpoint chosen is representative of a wide variety of endpoints that will adversely impact both the individual and the population since we must consider that any mutational event will lead to a reduced selective advantage. From a standpoint of negatively impacting the individual, it is small compensation to the couple involved to be informed that they are a "high risk pregnancy" couple, but that they may not be at a higher risk when one considers the integrity of successful term pregnancies.

4.2 RADIATION INDUCED MUTATIONS IN OOCYTES.

The in vitro methodology detects dominant lethal mutations that are induced by x-radiation in oocytes. These data differ somewhat from those generated by an in vivo approach. In those studies, the rate of dominant lethal mutations increased in a curvilinear fashion with increased dose. The shape of the mutation-induction curve was consistent with lesions induced by two-hit processes. If applied to risk assessment in humans, these data would support a prediction of few induced mutations at low doses and then a sharply increasing rate at higher doses. The dominant lethal data from the in vivo studies are not in agreement with cytogenetic data for chromosome aberration induction. Chromosome aberrations are induced in a linear fashion with increasing dose of x-radiation. If dominant lethality and chromosome aberrations are causally related, then these observations suggest that some detectable aberrations do not cause dominant lethal mutations. Since it is unlikely that current cytogenetic methods detect all (or even most) chromosome aberrations, it seems unlikely that the dominant lethal rate should be so much less than the rate of chromosome aberrations.

It might be argued that the in vivo dominant lethal data, gives an artificially low result at low doses because the contribution to overall dominant lethality by preimplantation losses is disregarded. The in vitro data do not support this argument because the rate of successful development to the blastocyst stage (and therefore to an implantation stage in vivo) was not lower in oocytes that had received less than about 2.0 Gy. Thus, at low doses, the in vivo and in vitro approaches should assess development to about the same efficiency. The in vivo data however may be adversely affected by embryo loss due to radiation-induced super ovulation and subsequent competition for implantation sites, especially at doses greater than 2.0 Gy. In this scenario the rising portion of the dose response curve may be indicative of an artificially high embryo loss due to a mechanism other than dominant lethality. The fertilization data in the present study indicate that the initial union of spermatozoa and oocyte is not affected by radiation to the oocyte.

The mouse model chosen here has certain limitations when one tries to apply it to risk assessment and setting exposure standards. It has already been mentioned that the mouse oocyte in the primary resting stages is extremely sensitive to radiation-induced cell killing whereas the human oocyte is resistant (61,65,88). By judicious selection of the stage irradiated and by limiting the normal variation in oocyte development by artificially timing ovulation, it was hoped that the mouse oocyte stage investigated would be representative of human oocyte. The criticism that there is an adverse or artifactual complication due to the method of induced ovulation cannot be answered unless a protocol is used that reduces the precision of oocyte selection that the technique affords.

The in vitro methodology results suggest that dominant lethal mutations are induced linearly with increasing dose and that the rate of induction decreases with higher doses. Transforming the date into a dominant lethal mutation rate [= -ln (1 - mutant rate)] results in a linear increase over the range tested. Thus it appears that dominant lethal mutations are wasted in oocytes that already have a dominant lethal mutation rather than there being repair or recovery at the higher doses. The shape of the induction curve indicates that there is no threshold for mutagenesis in oocytes.

There is some evidence that the resting-stage oocyte can repair damage induced by UV or other stresses (100). Because of the inherent differences between

the mouse model and the human situation (88,89), one cannot rule out a potential modification of the net dominant lethal rate if repair was active between the time of irradiation and ovulation. In setting exposure standards, the prudent approach is to disregard potential repair, an interpretation that is consistent with linear induction kinetics. Again, in both the female mouse and the female human, the number of oocytes is fixed soon after birth and there is no possibility to recover those killed by the radiation, and little hope of repairing those carrying cytogenetic damage.

4.3 DOSE MODIFICATION OF RADIATION INDUCED MUTATIONS BY DRUGS.

The usual way in which the action of radiation is modified is by the addition during the exposure of a compound that in some ways modifies the endogenous sulphydryl content. For cell survival, hypoxic cells are sensitized when compounds that reduce sulphydryl such as N-ethylmaleimide or buthionine sulfoximine are present. Euoxic cells are sensitized by the addition of sulphydryl containing agents such as mercaptoethylamine. Because germ cells are euoxic, any strategy to modify the yield of radiation-induced mutations must be based on a euoxic type response. Thus, cells might be protected from mutagenesis by the addition of sulphydryl agents or by selective elimination of mutated cells relative to undamaged cells. To increase mutagenesis, one must attempt to use approaches that rely on other aspects of mutation induction such as specificity during the cell cycle.

Sigestad et al.(99) have reported that the addition of the radioprotector Wr-2721 reduces the yield of mutants at the HGPRT locus in cultured cells. A time course was described which limited the activity to some hours before, during,and immediately after irradiation by either gamma radiation or Janus neutrons. This result is encouraging for work in germ cells where it has yet to be tested. Other sulphydryl compounds such as 2-aminoethylisothiouroniumbromide hydrobromide have had some success in protecting germ cells (79,90). Because the majority of lesions induced by densely ionizing radiations result in damage at the chromosome level, any strategy must be directed primarily at that mechanism.

It is possible theoretically to reduce the mutational yield by changing a potentially mutagenic lesion into a lethal one thereby eliminating, selectively,

mutated cells. From the results presented here, both in male and female germ cells exposed to densely ionizing radiation, it must be kept in mind that the type of lesion induced is the same for both mutagenic and lethal lesions. The strategy to selectively reduce mutant yield does so only at the increased risk of inducing other mutations. Although the data are very incomplete, the results in male germ cells suggest that the probability of inducing a lethal lesion is higher than the probability of inducing a mutagenic lesion at any given dose. This is seen in two aspects: (1) The fertility rate is depressed to extents that far exceed the mutation rates (if infertility reflects killing of more than 90% of the cells at risk; and, (2), the mutant rate is higher in cells that do not divide and therefore do not have the opportunity to express lethal lesions than it is in cells that must divide to finish spermatogenesis. Therefore cell division acts to remove potentially mutated cells and it should be possible to modify lesions that would be mutagenic into lesions that are lethal at cell division.

In the clinic it has been observed that the rate of secondary neoplasia is lower in children who received radiation and actinomycin D in their treatment than in children who received only radiation (80). A similar response was found in cells given both radiation and Actinomycin D and has been termed "mutational extinction" (81). The timing of the response in cells is such that selective killing of mutated cells may not be occurring but rather a generalized killing of both mutated and non-mutated cells in a specific, sensitive portion of the cell cycle is causing the response (101). For example, the alkylating agent nitrogen mustard (mechlorethamine) is mutagenic in all phases of the cell cycle but selectively kills cells before they have replicated their DNA (102). X-radiation also preferentially kills cells before they have replicated their DNA. Therefore use of these two agents sequentially might have little effect because there is a single sensitive population at risk. In contrast, vincristine kills cells in the phase of the cycle after they have replicated their DNA (102). Sequential administration of mechlorethamine and vincristine should influence the mutational yield depending on the order of administration and the timing. A strategy based on this action requires that the administration of the two agents be within one cell cycle and might therefore have limited application to field exposures.

Chemical agents may also selectively kill cycling or non-cycling cells. This was the strategy explored in the studies with dose modification in this contract. The

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data suggest that pretreatment with a combination of mechlorethamine and procarbazine reduces the yield of radiation induced mutations from 12.4% without pretreatment to less than controls. These data are complicated by the fact that the chemical treatment alone was not mutagenic in this trial. Other work in this laboratory indicate that when mechlorethamine is used in conjunction with procarbazine and the mechlorethamine is given first, significant mutagenesis results (Goldstein, in press). In contrast, when procarbazine precedes mechlorethamine, no mutagenesis was detected. Thus, while the data certainly suggest that pretreatment with these two chemotherapeutic drugs markedly decreases the mutant yield, more work will have to be done to verify the result.

Another approach that may prove worthwhile is to have the chemical follow the irradiation within a short time, preferably while cells mutated in the time preceding DNA synthesis have not progressed to the DNA synthetic stage. An underlying assumption in this strategy is that dominant lethality is primarily the result of chromosome aberrations, rather than chromatid aberrations. I do not make the distinction between structural and numerical aberrations in this context since both essentially arise from related mechanisms. The data support such a conclusion since the yield of micronuclei in this and other studies is always less than the yield of dominant lethal mutations. Other work in this laboratory indicate that varying the time of treatment between the application of two mutagens can alter the yield of dominant lethal mutations (Goldstein, submitted). Whether this occurs by mutational extinction or by non-related mechanisms is unclear.

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SECTION 5

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